THE EFFECT OF CARBON DIOXIDE ON ANAEROBIC SPOREFORMERS

BY

CARROLL EUGENE HEIST

B.S., Muhlenberg College, 1930
M.S., University of Illinois, 1947

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BACTERIOLOGY
IN THE GRADUATE COLLEGE OF THE
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R. UNIV. BIBLIOTHEEK LEIDEN

THE EFFECT OF CARBON DIOXIDE ON ANAEROBIC SPOREFORMERS

Much controversy is evident in the literature on the action of high concentrations of carbon dioxide on the bacterial cell. Very little attention has been given the anaerobic sporeformers. This investigation is an effort to determine the effects of carbon dioxide on *Clostridium botulinum* type A, strain 701; type B, strain 791 and type C, strain 827. *Clostridium sporogenes* strain 877 and *Clostridium putrefaciens* strain 936 were likewise investigated but to a lesser extent.

The objects of these studies were:

1. To determine whether high concentrations of carbon dioxide stimulate or inhibit germination of heat treated spores, multiplication of vegetative cells and spore formation.

2. To investigate if the mode of action was influenced by hydrogen-ion concentration or the direct results of carbon dioxide.

Experimental Procedure

Techniques — The air was exhausted from a sealed liter flask with a vacuum pump. The flask contained 400 ml. of buffered pork extract broth which was saturated at a given temperature with 1.0, 0.7, 0.4 and 0.2 atmospheres of carbon dioxide. After the process of saturation was completed the pressure in the flask was brought to 800 mm. of mercury with nitrogen gas from a commercial cylinder. The pH of the carbon dioxide treated samples were determined electrometrically. Two nitrogen samples were prepared, one with a pH equivalent to, or less than, the carbonated sample saturated with one atmosphere of carbon dioxide, the other nitrogen control retained the original pH of the medium as determined before carbonation took place. The flasks were then seeded with heat treated spores through a pipette inserted into a rubber stopper, fitted into the mouth of the flask. The number

of spores inoculated were determined by a viable count.

Spores were separated from vegetative cells by exposing samples to the temperature of 70°C. for 15 minutes in a hot water bath. All dilutions were done anaerobically, since aerobic dilutions of actively growing cells did not give representative counts. One ml. portions of the desired dilution were transfered from the diluting apparatus into spore counting tubes. The titratable acidity of each sample was determined by titrating 10 ml. samples against a N/10 sodium hydroxide solution with phenolphthalein as an indicator. At the same time the pH of the samples were checked electrometrically.

The number of viable cells and spores present at a given time in the carbonated samples was compared with the acidified nitrogen controls, with the same or a lower pH. This nitrogen control was acidified with dilute hydrochloric acid to the desired pH. Since the nitrogen control and the carbonated samples had the same pH, stimulation or inhibition as determined by viable counts were attributed to the direct action of carbon dioxide or changes in the medium brought about by carbonation, and not the result of hydrogen-ion concentration.

The second or normal nitrogen control retained the original pH of the medium before carbon dioxide gas was added. The difference in the number of viable cells in the normal nitrogen control and the carbonated samples indicated the effects of carbon dioxide itself as well as the effect of pH as a result of carbonation.

Experiment No. 1.

Results — With a small inoculum of heat treated spores, pork extract broth saturated with 1.0, 0.4 and 0.2 atmospheres of carbon dioxide at 20°C. and incubated at the constant temperature of 20°C. completely inhibited germination, growth, multiplication and toxin formation of *Clostridium botulinum* types A, B, and C spores for 22 days.

After incubation for 22 days at 20°C. the carbon dioxide was exhausted and replaced with nitrogen. Incubation for 10 days at 37.5°C. did not stimulate germination of spores. The normal nitrogen control produced viable cells and toxins at 20°C.

Conclusion — These results seem to indicate that the inhibitory action of carbon dioxide is not entirely the result of lowered pH, but depends, to some extent, upon the direct action of carbon dioxide. One would not expect a pH of 6.4 to inhibit germination of spores at 37.5°C.

Experiment No. 2.

Results — A series of 18 flasks were inoculated with types A, B, and C *Clostridium botulinum* heat treated spores, saturated at 20°C. with 1.0, 0.7, 0.4 and 0.2 atmospheres of carbon dioxide and incubated at room temperature. One nitrogen control was acidified with dilute hydrochloric acid to a pH of 5.70, the second nitrogen control retained the original pH of 7.30. There was little or no difference between the total number of cells in the nitrogen control, pH 7.30, and the test samples saturated with 0.2 atmospheres of carbon dioxide. Sporulation was somewhat inhibited in the 20% carbonated samples. The nitrogen control with a pH of 5.70 showed some turbidity and a few demonstratable viable cells, and slight toxin production. No spores could be detected at any time. None of the other carbon dioxide treated samples showed turbidity or growth. However, type C spores treated with a 40% atmosphere of carbon dioxide produced some toxin.

Conclusion — Carbon dioxide as demonstrated under the conditions of this experiment showed inhibitory action other than those resulting from changes in pH at room temperature.

Experiment No. 3.

Results — Pork extract broth was saturated at 20°C. with a 70% atmosphere of carbon dioxide, incubated at 20°C. and seeded with a heavy inoculum of *Clostridium botulinum* types

A, B, and C heat treated spores. There was definite growth and toxin production. However, type B with the same number of spores, saturated with 1 atmosphere of carbon dioxide demonstrated neither growth nor toxin production. The nitrogen control with a similar pH was not inhibited by the hydrogen-ion concentration.

Conclusion — These experimental results indicated that carbon dioxide is less effective as an inhibitory agent with a heavy inoculum.

Experiment No. 4.

Results — When *Clostridium botulinum* types A, B, and C spores were inoculated into pork extract broth saturated with one atmosphere of carbon dioxide at 24 and 27°C., respectively, incubated at room temperature, there was a slight inhibitory action. The final number of viable cells in both the nitrogen control and the carbonated samples were approximately the same. Spore formation was somewhat inhibited in the carbon dioxide treated specimens.

Conclusions — These results indicate that the initial temperature which controls the solubility of carbon dioxide influences the bacteriostatic effects of carbon dioxide.

Experiment No. 5.

Results — *Clostridium sporogenes,* strain 877 and *Clostridium putrefaciens,* strain 936, heat treated spores were inoculated into pork extract broth saturated at 20°C. with 1.0, 0.7, 0.4 and 0.2 atmospheres of carbon dioxide and incubated at room temperature. Two nitrogen controls were prepared, respectively, for each species, one with a pH of 7.20, the other was acidified with hydrochloric acid to a pH of 5.95. After five days incubation the nitrogen controls produced twice as many Clostridium spores as the highest carbonated spore producer. Atmospheres of 100 and 70% carbon dioxide brought about complete growth inhibition. The hydrogen-ion concentration of the acidified nitrogen control did not seem

to interfere with growth or spore formation of *Clostridium sporogenes*. *Clostridium putrefaciens* showed only a slight initial inhibitory reaction to these high concentrations of carbon dioxide while the nitrogen control with a pH of 5.95 developed no viable cells. When *Clostridium sporogenes* was subjected to the same concentrations of carbon dioxide and incubated at 20°C., a 0.2 atmosphere of carbon dioxide was sufficient to inhibit growth. The temperature was increased respectively to 23 and 37.5°C. for approximately 30 days. The inhibitory action of carbon dioxide persisted in spite of the elevated temperature.

Conclusions — Carbon dioxide exerted a definite inhibitory action on *Clostridium sporogenes*, its effectiveness was definitely increased at lower temperatures. It appears that the spores exposed for an indefinite period of time to high concentration of carbon dioxide in pork extract broth at 20°C. will not recover once they are inactivated. This same phenomenon has repeatedly been demonstrated with *Clostridium botulinum* spores types A, B, and C. *Clostridium putrefaciens* showed a marked tolerance to high concentrations of carbon dioxide. The experimental data indicated that certain spore formers showed greater tolerance to carbon dioxide than others.

The same three strains of *Clostridium botulinum* spores were exposed respectively to carbon dioxide atmospheres of 10 to 20, 35 to 45, 75 to 85 and 100% on pork extract agar, pH 7.4. Mixtures of carbon dioxide and nitrogen gas were allowed to flow over a series of flasks seeded with the heat treated spores. A nitrogen control series consisting of a similar number of inoculated flasks were exposed to an atmosphere of nitrogen gas. At various intervals of time, the last flask in each series was detached, a suspension of the surface growth was diluted anerobically and the number of cells present at a given time determined by viable count.

This technique furnished evidence of specific strain variation. A 75 to 85% tension of carbon dioxide inhibited

almost completely types A and B, while type C showed a favorable reaction to this concentration of carbon dioxide. One atmosphere of carbon dioxide inhibited completely types A and B; type C developed chalky white colonies. The carbon dioxide tolerance of type C strain 827 was so pronounced that it was easy to separate this strain from type A, 701, and type B, 791.

The author hopes to further investigate whether carbon dioxide tolerance is peculiar only to strain 827 studied in this experiment or a characteristic of type C.

VITA

Carroll Eugene Heist was born near Allentown, Pennsyslvania, on January 14, 1905. He attended the rural elementary school near Allentown, thereafter entered the Allentown Preparatory School and was graduated in 1926. He attended Muhlenberg College, Allentown, Pennsylvania, receiving a B. S. degree in 1930. In 1933, he completed the first two years in the study of medicine at Temple University, Philadelphia, Pennsylvania.

On June 13, 1941, he was drafted into the Army, was honorably discharged on November 16, 1944, as a Technical Sergeant in charge of a Clinical Laboratory, to accept a commission in the Sanitary Corps. He spent a little over a year at the Halloran General Hospital, Staten Island, New York, in research on streptomycin and was discharged from active duty on January 7, 1946. In February of the same year he was admitted to the Graduate School of the University of Illinois, and received the M. S. degree in Bacteriology in February 1947.

He is a member of Sigma Xi, Society of American Bacteriologists, American Public Health Association, and Phi Chi Medical Fraternity.